IN THE UNITED STATED PATENT AND TRADEMARK OFFICE

Applicants: GIANNI Alessandro Massimo, CARLO STELLA Carmelo, COLOTTA Francesco

Serial No. 10/565,903

Filed : July 23, 2004

Title: PHARMACEUTICAL COMBINATION USEFUL FOR STEM CELLS MOBILIZATION

National Phase of the international Patent Application No.PCT/EP04/08245 filed on June 21, 2006

DECLARATION UNDER 37 CFR1.132

I, Alessandro M. GIANNI, being duly sworn depose and say that:

1. I am an Italian citizen residing at: Milano (Italy)
2. I am familiar with the English language.
3. I graduated in: Medicine in the academic year: 1968 at the University of Milano
4. I am author of more than 450 scientifical publications.
5. Previous job experiences:

   1974/1976 - Center for Cancer Research, MIT (Cambridge, Massachussetts, USA), Research Associate

   1992/1998 - University of Milano, Associate Professor in Medical Oncology

6. Actual job :

   Director, Medical Oncology Division, Fondazione IRCCS Istituto Nazionale Tumori, Milano

   Science Vice-Director for Clinical Science, Fondazione IRCCS Istituto Nazionale dei Tumori, Milano

   Full Professor of Medical Oncology, University of Milano

7. I further declare what follows

To the best of my knowledge and experience, as also pointed out in the Specification, the administration of adenoviral vectors expressing a growth factor presents several major differences from the direct injection of the same purified factor, and therefore cannot at all be predictive of the effects triggered by the growth factor when this is administered according to the modalities used in the clinical settings.

In fact, it is well known that the adenoviral system is a "facilitated" system of administration of a growth factor compared to administration of the same factor as such. In fact, in AdPLGF the adenovirus acts as a selective delivery system for PlGF, that is able to transport PlGF cDNA into specific target cells and to induce the expression of the growth factor. This facilitating effect exerted by the adenovirus is, on the contrary, not present in clinical setting, when PlGF is administered as such. The above difference is also reflected in the different pharmacokinetic profile obtained by the two types of administrations. In particular, while the injection of an adenovector expressing PlGF allows long-lasting and elevated PlGF plasma levels, injection of the recombinant protein as such only elicits transient effects.

In view of the above, while the lack of an effect on mobilisation with AdPLGF would have certainly been suggestive of the lack of an effect also with PlGF as such, the contrary could be assumed.

This is indeed confirmed by comparing the results reported in Hattori with AdPlGf and those reported in the present Specification with PlGF *as such.*

In details, in Hattori a single intravenous administration of the adenoviral vector expressing the recombinant human PlGF is able:

- to induce a 20 fold increase of CFU-S/10^5PBMCs (see page 845 lines 4-6 left column) as compared with control mice three days after adenoviral infection (figure 4c),
- to induce a 14 fold increase in the total number of circulating progenitor cells in AD-PlGF treated mice if compared with Ad-Null (progenitors cells capable of forming CFU colonies) (CF-GEMM, CFU-M, BFU-E) in BALB/c mice three days after a single intravenous administration (see figure 4b and comments thereof in the legenda of the figure).

On the contrary, the results of following examples carried out under my own responsibility demonstrate that:

- Example 2 of the present application, reports that mice treated intraperitoneally with 5 μg /day for 5 days with recombinant mouse PlGF (rmPlGF) show almost the same mean frequency (8±1 CFC/10^5MNCs) of circulating CFCs as the control mice (8±3 CFC/10^5MNCs) treated with PBS/MSA (table 2)
- Example 3 of the present application reports that mice treated intraperitoneally with 5 μg /day for 5 days with recombinant mouse PlGF (rmPlGF) exhibit almost the same absolute number (96 ± 13) of circulating CFCs of control mice (81 ± 75).(see table 3)
- Example 6 of the present application reports that mice treated intraperitoneally with 10 μg/day recombinant human PlGF (rhPlGF) for 5 days exhibit almost the same mean frequency (10 ± 4) CFCs of control mice (8 ± 3).(see table 6)
- Example 7 of the present application reports that mice treated with mice treated intraperitoneally with 10 μg/day recombinant human PlGF (rhPlGF) for 5 days exhibit almost the same mean absolute number (82 ± 64) of circulating CFCs of control mice(81 ± 75).(see table 7)

The above results clearly highlight that, when recombinant mouse or even human PlGF (namely the same type of PlGF expressed by the Adenovirus of Hattori) is administered as such (in

other words it is a purified factor) as the sole active ingredient, contrary to what happens in the case of Hattori's AdPlGF, it is completely unable to increase blood cells mobilization.

In vivo injection of an adenoviral vector might induce the release of inflammatory cytokines cooperating with PlGF in inducing PBPC mobilization. Under our experimental conditions, rhPlGF-1 administration was associated with a modest, if any, increase of plasma MMP-9, further corroborating the lack of any mobilizing activity of rhPlGF-1 alone.

As discussed above, the lack of white blood cell and progenitor cell release into the bloodstream under rhPlGF-1 as such is in contrast with Hattori's adenoviral system is not to be ascribed to the different route of administration of these two substances (intravenous for AdPlGF, intraperitoneal for PlGF), but it is due to differences in pharmacokinetic parameters consequent to the two different forms in which PlGF is administered.

Both administration routes are indeed parenteral injections and, as also reported in enclosure 1, IP injection is predominantly used in animal testing for the administration of systemic drugs and fluids due to the ease of administration as compared with other parenteral methods.

Therefore an intraperitoneal administration and an intravenous administration in BALB-c mice have a comparable behaviour in term of bioavailability of the active substance.

In view of the foregoing, the striking difference between AdPlGF and PlGF in the above discussed experiments cannot be attributed to the different route of administration, but rather to the intrinsic nature of the substances involved in the two experiments.

In view of the foregoing results, from Hattori disclosing that the adenoviral vector expressing PlGF, when administered alone, is able to increase in a high degree the blood cell mobilization, I would not have been certainly induced to add PlGF as such to GCSF with the aim of overcoming Robinson's drawbacks, i.e. in order to increase blood cells mobilisation since :

a) I am aware of the major differences existing between the administration of an adenoviral vector expressing a growth factor, like that expressing PlGF disclosed by Hattori and that of the growth factor as such, in the specific PlGF, these differences are also supported by the experimental results above evidenced, which clearly demonstrate that a single intravenous

administration of AdPlGF, produces a 20 fold increase of CFU-S/10^5PBMCs, a 14 fold increase in the total number of circulating progenitor cells, whereas PlGF as such, when administered alone, is able neither to increase the frequency nor the total number of circulating blood cells.

b) The above difference of behaviour in terms of cells mobilization cannot be attributed to the different type of administrations of these two active ingredient, which, according to Hattori, is a single intravenous injection to BALB-C-MICE, whereas in the examples reported in the Specification are repeated intraperitoneal injections to the same type of mice, but rather to the intrinsic nature of the substances involved in the experiments.

In details, it is quite surprising and unexpected, from the combination of Robinson with Hattori teachings, having found that:

- as reported in Example 2, while PlGF administered alone is inactive, the same, when administered intraperitoneally at the same dosage (5μg/day) and with the same modalities (for 5 days) in association with G-CSF, is able to increase the frequency of CFCs of from about 130 (the mobilization obtained with the sole G-CSF) to about 180, in other words a mean frequency of CFCs of about 1.4 folds that obtained after administration of the sole G-CSF.
- as reported in example 3, while PlGF administered alone is inactive, the same, when administered intraperitoneally at the same dosage (5μg/day) and with the same modalities (for 5 days) in association with G-CSF, is able to increase the absolute number of circulating CFCs in mice of from a mean value of 2,977± 1,126 obtained with administration of the GCSF alone to a mean value of from 6,015±3,674, in other words a number of circulating CFCs in mice of 2 folds that obtained with the sole G-CSF.
- As reported in example 6, while recombinant human PlGF administered alone is inactive, the same, when administered intraperitoneally at the same dosage (10μg/day) and with the same modalities (for 5 days) in association with G-CSF, is able to increase the mean frequency of CFCs of from about 82±29 obtained with the sole G-CSF, to about 256±77, in other words to a mean frequency of CFCs of

more than three folds (3.12) that obtained after administration of the sole G-CSF (see example 6). Moreover as demonstrated in the same example rhPlGF, also when administered at a dosage of only 5 μg/day in association with G-CSF is able to increase the mean frequency of CFCs of from 82 obtained with administration of the GCSF alone to about 224±14, therefore to a mean frequency of almost three folds (2.73) that obtained with rhGCSF alone.

- as reported in example 7, while rhPlGF administered alone is inactive, the same, when administered intraperitoneally at the same dosage (10μg/day) and with the same modalities (for 5 days) in association with G-CSF, is able to increase the absolute number of circulating CFCs in mice of from a mean value of 2,977± 1,126 obtained with administration of the GCSF alone, to a mean value of from 12,122±2,788, in other words to a mean absolute number of circulating blood cells 4 folds that obtained with the sole G-CSF. Moreover as demonstrated in the same example rhPlGF, also when administered at a dosage of only 5 μg/day in association with G-CSF is able to increase the absolute number of circulating CFCs in mice of from a mean value of 2,977± 1,126 to a mean value of 9,435 ± 1,906 , in other words to a mean value of absolute number of circulating blood cells of about two folds (2.17) if compared to those obtained with the sole G-CSF.

Surprisingly, while the administration of PlGF alone did not influence MMP-9 plasma levels, the combined rhPlGF-1/rhG-CSF administration had an additive effect on MMP-9 levels, suggesting that this protease might be critically involved in the molecular mechanism(s) underlying the synergism of rhPlGF-1 and rhG-CSF. Since MMP-9 plays a key role in PBPC mobilization by either releasing sKitL or degrading SDF-1, it is possible to hypothesize that the release of proteases by either stromal cells or neutrophils accumulating into the bone marrow during rhG-CSF administration is surprisingly significantly enhanced by the concomitant rhPlGF-1/rhG-CSF injection, thus resulting in a synergistic effect on hematopoietic mobilization, as discussed above.

I finally declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that such wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such wilful false statements may jeopardise the validity of the applications or any patents or re-examination certificate issued thereon.

Milano, November 12, 2009

Alessandro M. Gianni, M.D.

ENCL. 1 : Intraperitoneal injection downloaded from Wikipedia